Interaction of neuroleptic drugs with a synthetic calcium-binding peptide analog of site III of rabbit skeletal troponin C

Phenothiazine selective binding

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The effects of nine drugs on the CD spectra of a synthetic calcium binding analog of site III of rabbit skeletal troponin C, can generally be divided into 3 groups: (1) that consisting of haloperidol, benperidol, molindone and promethazine, had no effect on the CD spectrum or calcium sensitivity of the apopeptide; (2) that composed of structurally rigid thioxanthenes, induced CD-detectable structural change in the apopeptide but prevented Ca^{2+}-induced structural change; (3) that consisting of chlorpromazine, trifluoperazine and fluphenazine, induced structural change in the peptide but had no effect on the Ca^{2+}-induced structural change.

Circular dichroism *Drug binding* *Synthetic peptide* *Troponin C*

1. INTRODUCTION

Following the discovery of calmodulin (CaM), a small acidic protein capable of activating both cAMP dependent phosphodiesterase [1,2] and adenylate cyclase [3–7] in a calcium-dependent manner, Levin and Weiss demonstrated that antipsychotic drugs inhibited the activation of cAMP phosphodiesterase [8]. Later, these same authors demonstrated that the phenothiazine antipsychotic trifluoperazine binds to calmodulin and troponin C in the presence of calcium [9,10]. Although the pharmacological significance of such interactions with calmodulin remains a controversial topic [8–14], it is clear that these compounds share structural features (i.e., positively charged and hydrophobic domains) present on peptides and proteins known to interact with CaM and troponin C [15–21]. Cyanogen bromide fragments containing site III of bovine brain CaM [22] and site III of rabbit skeletal troponin C [23] have been shown to interact with phenothiazines. One of the drug binding sites was restricted to an 8 amino acid long region of troponin C (residues 95–102) found to be highly conserved in CaM (residues 85–92) [23]. We have synthesized a 34-residue analog of site III of rabbit skeletal troponin C (region 90–123) containing both the proposed trifluoperazine-binding site and a high affinity calcium-binding region [24] and made use of this model peptide to examine the effect of a variety of neuroleptic drugs on the secondary structure and the Ca^{2+}-binding properties of this calcium-binding unit.

Abbreviations: CaM, calmodulin; cAMP, 3′-5′ cyclic adenosine monophosphate; CD, circular dichroism; EGTA, ethylene glycol bis (β-aminoethyl ether); *N,N,N′,N′*-tetraacetic acid; MOPS, 3-(*N*-morpholino) propanesulfonic acid; TnC, troponin C

2. MATERIALS AND METHODS

All chemicals and solvents are reagent grade unless otherwise stated. The drugs were generous

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gifts from the following companies: trifluoperazine (Smith Kline and French, Montreal, Quebec); *cis*- and *trans*-thiothixene (Pfizer Inc., Groton CT); benperidol, Janssen Pharmaceutica (Beerse); haloperidol, McNeil Labs (Stouffville, Ontario); molindone, Endo Labs (Garden City NY); chlorpromazine and promethazine, Poulenc Ltd. (Montreal, Quebec); fluphenazine, E.R. Squibb and Sons Ltd. (Montreal, Quebec).

2.1. *Peptide synthesis and purification*

The 34-residue peptide was prepared by the automated solid-phase method, cleaved from the resin by anhydrous HF and purified by DEAE–Sephacel and hydroxyapatite chromatography as in [24].

2.2. *Preparation of samples*

The buffer system chosen for the circular dichroism (CD) studies was 100 mM MOPS (pH 6.0), 50 mM KCl, 5 mM EGTA. Ca^{2+} free buffers were prepared from deionized distilled water which had been treated with Chelex resin. The peptide was dissolved in the above buffer and dialyzed overnight against the same buffer to remove traces of calcium. The appropriate drug was dissolved in the above buffer, then the peptide and drug solutions were mixed to produce a drug:peptide ratio of 2.3:1 and the CD spectra were determined. The drug solutions were freshly prepared and maintained in the dark throughout the circular dichroism experiments. To avoid contamination of solutions with Ca^{2+} from laboratory glassware, Nalgene beakers, test tubes and solution bottles were used throughout the study. Concentration of calcium solutions used in the study were determined by titration with a standard EGTA solution using murexide as an indicator [25]. Quantities of peptide were determined from either amino acid analysis or from measuring the absorbance of the peptide dissolved in 100 mM MOPS (pH 6.0), 50 mM KCl and 5 mM EGTA using an ϵ_{275} of $1.708 \times 10^3\ M^{-1}.cm^{-1}$ determined using quantitative amino acid analysis.

2.3. *Circular dichroism measurements*

CD measurements were made on a Cary 60 spectropolarimeter with a 6001 CD attachment as in [24]. The concentration of free Ca^{2+} in solution was controlled by an EGTA-containing buffer and calculated using the computer program in [26] as described in [24]. Calcium association constants were determined by a non-linear curve-fitting iterative procedure in [24].

3. RESULTS AND DISCUSSION

A 34-amino acid residue peptide analog of calcium binding site III of troponin C and, by sequence homology, site III of calmodulin (fig.1) has been tested for interaction with representatives of 4 different classes of neuroleptic drugs. The butyrophenones, represented by benperidol and haloperidol could not induce any CD-detectable structural change in the peptide up to a concentration rate of 2.3:1, drug:peptide (fig.2). Earlier investigations [11,21] had indicated that haloperidol did not bind to calmodulin as strongly as the phenothiazines. Thus the hydrophobic character of these drugs does not represent the sole criterion for a drug–peptide interaction.

Both of the thioxanthenes, *cis*- and *trans*-thiothixene, induced a small amount of structure in the peptide and this class was the only class tested that prevented the peptide from responding normally to the addition of calcium; i.e., with an increase in CD-detectable structural change (fig.2). The possibility exists that the inflexibility of the sidechains linked to the tricyclic ring structure by a double bond may have some effect on the ability of the drug–peptide complex to bind calcium.

A third class tested, the dihydroinolones, a non-classical group of neuroleptics represented by molindone [27], also failed to induce structural change in the peptide and did not affect the subsequent structural change induced by addition of calcium (fig.2). Finally, the phenothiazines represented by promethazine, chlorpromazine, trifluoperazine and fluphenazine induced structural change in the peptide in a manner corresponding to their in vitro ability to inhibit phosphodiesterase activation by calmodulin [11,21], but had no effect on the Ca^{2+}-induced structural change (fig.2). Note that the calcium-binding constant of this model peptide is equal to $2.6 \times 10^5\ M^{-1}$ in the absence of drug [24]. Except for the case of the thioxanthene isomers, the binding or presence of these drugs did not alter the calcium-binding property of the synthetic analog.

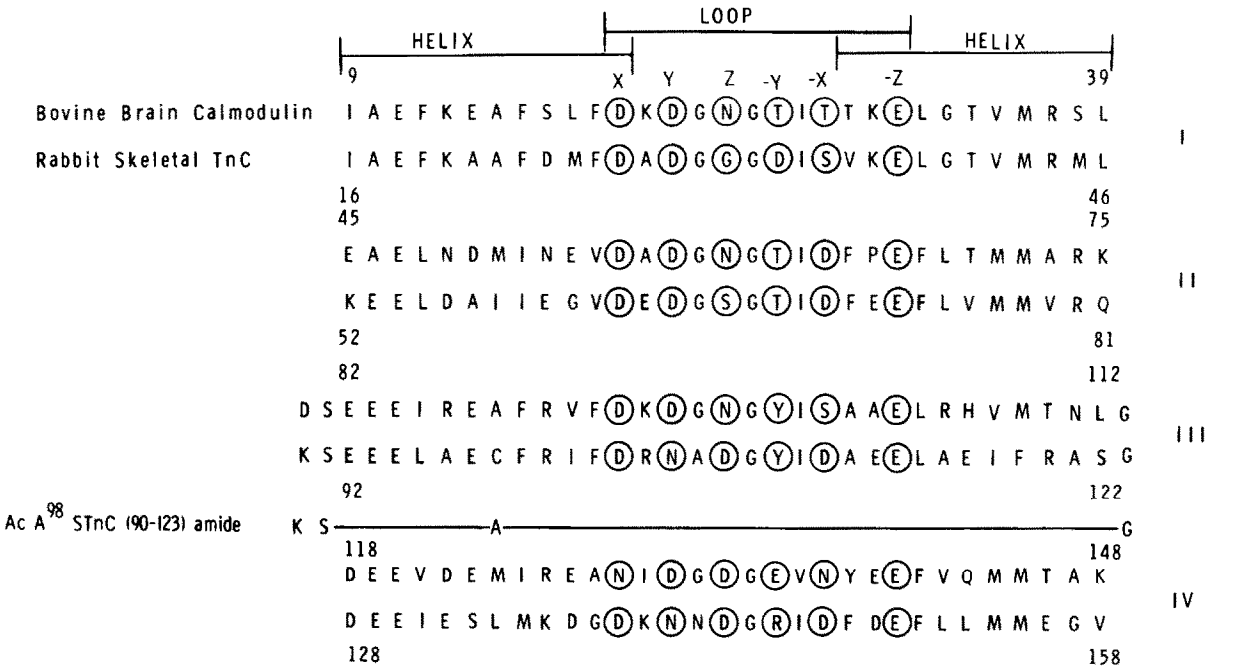

Fig.1. Amino acid sequence of the calcium-binding sites in bovine brain calmodulin, rabbit skeletal troponin C and the synthetic 34-residue peptide. The calcium binding sites are numbered I–IV. Bovine brain calmodulin is the upper sequence while rabbit skeletal troponin C is the lower sequence. The 34-residue peptide, AcA^{98} STnC (90–123) amide, is indicated directly below rabbit skeletal troponin C site III and has an identical sequence to this site with the exceptions indicated. The sequences comprising the helix–loop–helix regions are indicated by bars at the top of the figure. The 6 octahedral Ca^{2+} coordinating positions are designated $X, Y, Z, -Y, -X, -Z$. The amino acid residues in the corresponding Ca^{2+} coordinating positions are circled. Amino acid residues are designated using the one-letter code.

This observation is in agreement with proton magnetic resonance and circular dichroism results obtained for the binding of trifluoperazine to a cyanogen bromide fragment representing site III of rabbit skeletal troponin C [23].

In light of the interaction of calmodulin with a variety of peptides, proteins [18,19,28,29] and structurally related antipsychotics [11,21], it was concluded that all these structures share in common an hydrophobic and a positively charged domain [20,21]. The analysis of the trifluoperazine-binding site of site III of rabbit skeletal troponin C reveals that complementary hydrophobic and negatively charged centers exist and are optimally positioned when calcium induces the formation of an α-helical arrangement in the N-terminal region of the peptide [23,24].

Differences in ellipticity induced by phenothiazines on the model peptide can be rationalized in terms of the distance separating the positively charged domain and the aromatic moiety of the drug, as postulated in [21,23]. For instance, the positive charge on trifluoperazine can be associated with either of the nitrogens of the piperazine ring thus placing the charge at a distance of 4 to 7 bondlengths from the aromatic center. The same holds true for fluphenazine, but in the case of chlorpromazine and promethazine, 4 and 3 bondlengths respectively separate the two centers.

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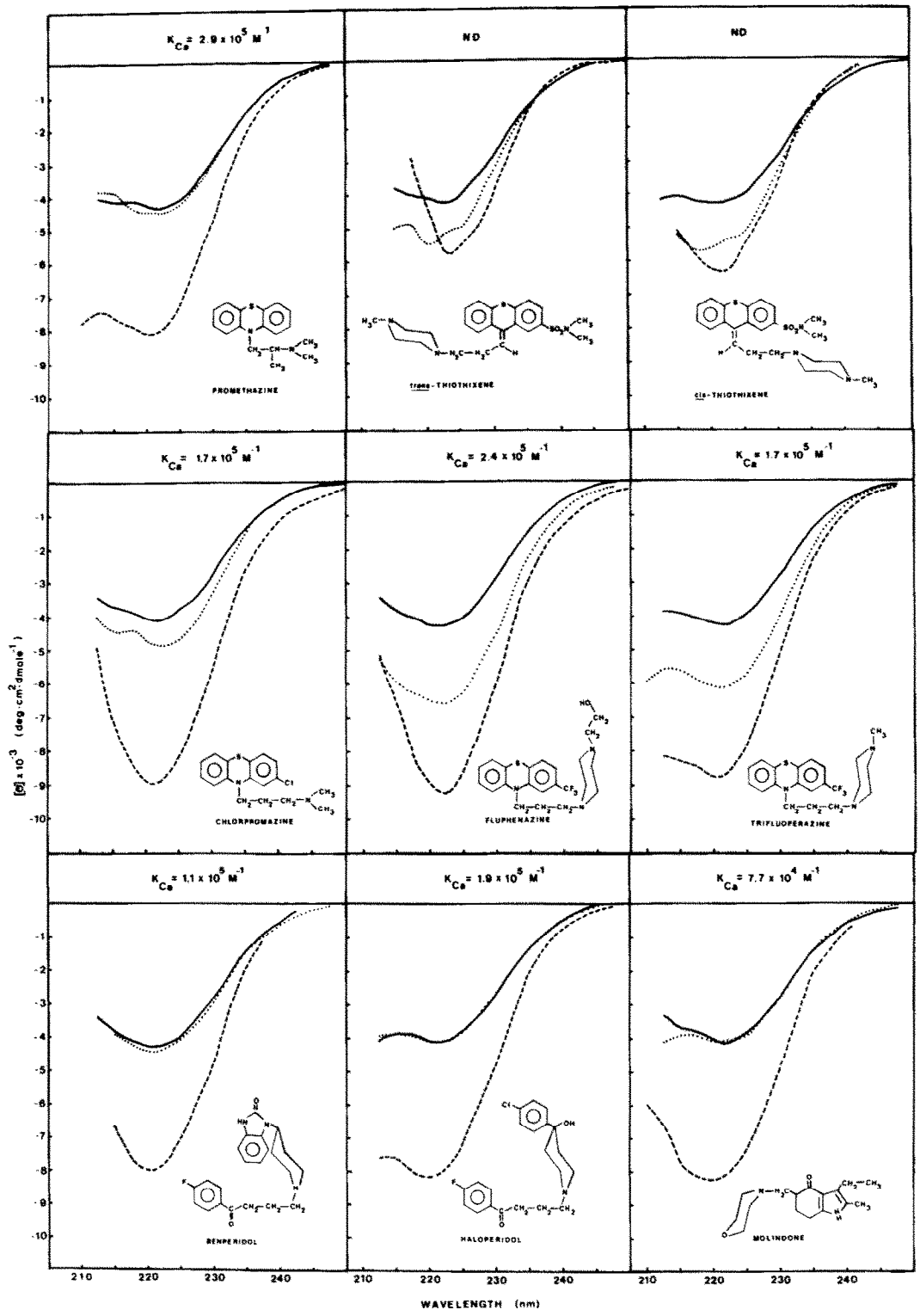
Fig.2. CD spectra: Drug and Ca^{2+}-induced CD spectra of the 34-residue peptide, native peptide (——), peptide + drug (···), peptide + drug + Ca^{2+} (– – –); peptide was 0.43 mM. The chemical structures of the various drugs are indicated beneath the corresponding spectrum. The K_{Ca}-values of the peptide in the presence of 1 mM drug are indicated at the top of the corresponding spectrum. The K_{Ca}-value of the peptide in the absence of any drug has been determined as $2.6 \times 10^5\ M^{-1}$ [24]. The CD spectrum for peptide + Ca^{2+} [24] is identical to that observed for peptide + Ca^{2+} + trifluoperazine. The experimental conditions are described in section 2; N.D., not determined.


K_{Ca} = 2.9 x 10^5 M^{-1}
ND
ND
PROMETHAZINE
trans-THIOTHIXENE
cis-THIOTHIXENE
K_{Ca} = 1.7 x 10^5 M^{-1}
K_{Ca} = 2.4 x 10^5 M^{-1}
K_{Ca} = 1.7 x 10^5 M^{-1}
CHLORPROMAZINE
FLUPHENAZINE
TRIFLUOPERAZINE
K_{Ca} = 1.1 x 10^5 M^{-1}
K_{Ca} = 1.9 x 10^5 M^{-1}
K_{Ca} = 7.7 x 10^4 M^{-1}
BENPERIDOL
HALOPERIDOL
MOLINDONE
[θ] x 10^{-3} (deg·cm²·dmole⁻¹)
WAVELENGTH (nm)
210
220
230
240

In conclusion, this work has pointed out the selectivity of a 34-residue synthetic peptide representing site III of rabbit skeletal troponin C for a particular class of neuroleptics and indicated its potential use as a phenothiazine selective binding region.

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REFERENCES

[1] Cheung, W.Y. (1970) Biochem. Biophys. Res. Commun. 38, 533–538.
[2] Kakiuchi, S. and Yamazaki, R. (1970) Biochem. Biophys. Res. Commun. 41, 1104–1110.
[3] Cheung, W.Y., Bradham, L.S., Lynch, T.J., Lin, Y.M. and Tallant, E.A. (1975) Biochem. Biophys. Res. Commun. 66, 1055–1062.
[4] Lynch, T.J., Tallant, E.A. and Cheung, W.Y. (1976) Biochem. Biophys. Res. Commun. 68, 616–625.
[5] Brostrom, C.O., Huang, Y.-C., Breckenridge, B.M. and Wolff, D.J. (1975) Proc. Natl. Acad. Sci. USA 72, 64–68.
[6] Brostrom, C.O., Brostrom, M.A. and Wolff, D.J. (1977) J. Biol. Chem. 252, 5677–5685.
[7] Bromstrom, M.A., Brostrom, C.O., Breckenridge, B.M. and Wolff, D.J. (1978) Adv. Cyclic Nucl. Res. 9, 85–99.
[8] Levin, R.M. and Weiss, B. (1976) Mol. Pharmacol. 12, 581–589.
[9] Levin, R.M. and Weiss, B. (1977) Mol. Pharmacol. 13, 690–697.
[10] Levin, R.M. and Weiss, B. (1978) Biochim. Biophys. Acta 540, 197–204.
[11] Levin, R.M. and Weiss, B. (1979) J. Pharmacol. Expt. Therapeut. 208, 454–459.
[12] Roufogalis, B.D. (1981) Biochem. Biophys. Res. Commun. 98, 607–613.
[13] Norman, J.A., Drummond, A.H. and Moser, P. (1979) Mol. Pharmacol. 16, 1089–1094.
[14] Landry, Y., Amellal, M. and Ruckstuhl (1981) Biochem. Pharmac. 30, 2031–2032.
[15] LaPorte, D.C., Wierman, B.M. and Storm, D.R. (1980) Biochemistry 19, 3814–3819.
[16] Tanaka, T. and Hidaka, H. (1980) J. Biol. Chem. 255, 11078–11080.
[17] Tanaka, T. and Hidaka, H. (1981) Biochem. Internat. 2, 71–75.
[18] Malencik, D.A. and Anderson, S. (1982) Biochemistry 21, 3480–3486.
[19] Sellinger-Barnette, M. and Weiss, B. (1982) Mol. Pharm. 21, 86–91.
[20] Weiss, B., Prozialeck, W.C. and Wallace, T.L. (1982) Biochem. Pharm. 31, 2217–2226.
[21] Prozialeck, W.C. and Weiss, B. (1982) J. Pharm. Expt. Therap. 222, 509–516.
[22] Head, J.F., Masure, R.H. and Kaminer, K. (1982) FEBS Lett. 137, 71–74.
[23] Gariépy, J. and Hodges, R.S. (1983) Biochemistry, in press.
[24] Reid, R.E., Gariépy, J., Saund, A.K. and Hodges, R.S. (1981) J. Biol. Chem. 256, 2742–2751.
[25] Blaedel, W.J. and Knight, H.T. (1954) Anal. Chem. 26, 743–746.
[26] Perrin, D.D. and Sayce, I.C. (1967) Talanta 14, 833–842.
[27] Kebabian, J.W. and Calne, D.B. (1979) Nature 277, 93–96.
[28] Grand, R.J.A. and Perry, S.V. (1980) Biochem. J. 189, 227–240.
[29] Itano, T., Itano, R. and Penniston, J.T. (1980) Biochem. J. 189, 455–459.